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Pathway profiles based on gene-set enrichment analysis in the honey bee *Apis mellifera* under brood rearing-suppressed conditions

Kyungmun Kim[a,1], Ju Hyeon Kim[a,1], Young Ho Kim[b], Seong-Eui Hong[c], Si Hyeock Lee[a,d,*]

[a] Department of Agricultural Biotechnology, Seoul National University, Seoul 151-921, Republic of Korea
[b] Department of Applied Biology, College of Ecology & Environmental Science, Kyungpook National University, Sangju, Gyeongbuk, Republic of Korea
[c] Theragen Etex, Bio Institute, Suwon, Gyeonggi-do, Republic of Korea
[d] Research Institute for Agriculture and Life Sciences, Seoul National University, Seoul, Republic of Korea

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ABSTRACT

Perturbation of normal behaviors in honey bee colonies by any external factor can immediately reduce the colony's capacity for brood rearing, which can eventually lead to colony collapse. To investigate the effects of brood-rearing suppression on the biology of honey bee workers, gene-set enrichment analysis of the transcriptomes of worker bees with or without suppressed brood rearing was performed. When brood rearing was suppressed, pathways associated with both protein degradation and synthesis were simultaneously over-represented in both nurses and foragers, and their overall pathway representation profiles resembled those of normal foragers and nurses, respectively. Thus, obstruction of normal labor induced over-representation in pathways related with reshaping of worker bee physiology, suggesting that transition of labor is physiologically reversible. In addition, some genes associated with the regulation of neuronal excitability, cellular and nutritional stress and aggressiveness were over-expressed under brood rearing suppression perhaps to manage in-hive stress under unfavorable conditions.

1. Introduction

Division of labor is one of the primary characteristics of eusocial insects such as the honey bee *Apis mellifera* [1]. While the reproductive queen is responsible for the production of offspring, different castes of sterile workers are involved in various tasks required for colony maintenance [2]. Task regulation of worker bees is primarily dependent on the age of workers, in which young workers perform in-hive tasks, such as nest building, cleaning, food storage and brood rearing, whereas old workers perform outdoor tasks, mainly foraging and colony defense [2]. This labor division mechanism is flexible and adjusted based on environmental and intra-colony factors [3,4]. Nevertheless, any environmental factors that exceed the threshold of colony flexibility, thereby disturbing normal labor division and behavioral maturation, can seriously disrupt nest homeostasis.

Global warming, which is the main aspect of climate change, has altered the timing of flower blooming and pollinator activity [5,6]. Such phenological shifts will eventually disrupt the temporal overlap between pollinators and their floral food resources, thereby reducing the availability of floral resources and the diet breadth of pollinators [5]. In the case of honey bee workers, which obtain the pollen and nectar solely from flowering plants, the synchronized timing of flowering and foraging is crucial with regard to brood care and colony maintenance. Therefore, the availability of pollen and nectar is the key limiting factor of the growth, development and productivity of honey bee colonies.

Climate change also leads to more frequent extreme weather, such as heavy rainfall, severe flooding, drought, and temperature extremes. Because extreme weather can strongly impair the foraging capability of a bee colony, these conditions could be disastrous for honey bees due to food shortage, particularly when during brood care [7]. Food availability inside and outside the hive also affects the division of labor and behavioral maturation of workers in a colony [8]. Since optimum growth rate and resource accumulation within the colony largely relies on division of labor [9], direct or indirect perturbation of normal behaviors (e.g., nursing and foraging) could immediately affect the colony's capacity for brood rearing.

Cooperative brood rearing is a central social behavior of honey bees based on the precise control of sociality, such as division of labor [10,11], communication between castes [12], and management of abiotic and biotic stress [13,14]. Therefore, if normal brood rearing in a honey bee colony is perturbed by external factors such as extreme

* Corresponding author at: Department of Agricultural Biotechnology, Seoul National University, Seoul 151-921, Republic of Korea.
E-mail address: shlee22@snu.ac.kr (S.H. Lee).
[1] These authors contributed equally to this work.

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weather, the entire colony would be placed under stressful conditions, resulting in the collapse of nest homeostasis. In our previous study, complete suppression of brood rearing activity was induced by placing active beehives in a screen tent, thereby blocking normal foraging activity during the active brood rearing season; the sealed brood area vanished completely within 56 days after screen tent installation [15]. When the screen tent was removed, however, brood rearing resumed rapidly and the sealed brood area steadily increased, demonstrating the restoration of normal social behavior [15]. Although a large body of information is available with regard to molecular and physiological aspects of labor division and behavioral maturation in honey bees [16–19], little is known regarding what physiological alterations occur when such normal brood rearing behaviors are sequentially suppressed.

In this study, we installed a screen tent over active beehives to simulate conditions under which workers could not forage normally and by which brood rearing would be artificially suppressed, such as during sudden torrential rain or unusually cold or hot weather. Then brood rearing was allowed to resume under a free foraging condition by removing the screen tent. To investigate physiological changes in worker bees (nurses and foragers) under these two brood rearing status conditions (normal brood rearing vs. suppression of brood rearing), transcriptomes of worker bee heads under the respective conditions were analyzed and compared. To better understand the biological processes associated with brood rearing status, gene-set enrichment analysis (GSEA) was performed, and the functional profiles of pathways under the influence of each condition were identified.

2. Materials and methods

2.1. Suppression and subsequent restoration of brood rearing in active colonies (screen tent experiment)

To suppress brood rearing by blocking foraging, four active bee hives were placed in a screen tent for 38 days in the summer (from June to July 2014) until brood rearing was completely suppressed, and then the screen was removed, allowing brood rearing to recover, as described previously [15]. To minimize age-related influences on gene expression under normal brood rearing and brood rearing-suppressed conditions, age-synchronized bee specimens were collected (see Fig. 1 for the collection scheme). On the 13th day after the placement of four active hives in the screen tent (Day 13), the youngest batch of newly hatched worker bees (500 individuals per hive) were marked with paint markers (Munhwa Co., Korea; different colors for different hives). Marked worker bees under the brood rearing-suppressed condition were collected 7 days (Day 20) and 25 days (Day 38) after marking as nurse bees (designated Net Nurse) and forager bees (designated Net Forager), respectively. The screen tent was removed on day 38 (after tent installment) (Day 0′) to restore brood rearing and all pre-marked bees were removed. After resuming brood rearing, the first batch of newly hatched worker bees was marked as before (Day 25′). The marked worker bees under the normal brood rearing condition were collected 7 days (Day 32′) and 25 days (Day 50′) after marking as nurse bees (designated Free Nurse) and forager bees (designated Free Forager), respectively. The collected marked bees were frozen with liquid nitrogen and stored at − 80 °C until RNA extraction and subsequent RNA-sequencing. Information of the samples were submitted to NCBI BioSample (SAMN06928121, Free Forager; SAMN06928122, Free Nurse; SAMN06928123, Net Forager; SAMN06928124, Net Nurse).

2.2. RNA extraction, library construction and sequencing

Five color-marked worker bees for each test group (i.e., Free Forager, Free Nurse, Net Forager and Net Nurse) were collected from colony number 1, and the individual batches of bees were pooled for RNA extraction. After the head parts were separated from the body under ice-cold conditions, they were immediately placed in 600 μl TRI reagent (MRC, USA) and homogenized with 0.5–2-mm beads (Nextadvance, USA) using a bullet blender (Nextadvance, USA). Total RNA was extracted according to the manufacturer's protocol. RNA purity was determined by assaying 1 μl of total RNA extract on a NanoDrop8000 spectrophotometer (Thermo, USA). Total RNA integrity was checked using an Agilent Technologies 2100 Bioanalyzer (Agilent Technologies, CA) with an RNA Integrity Number (RIN) value. mRNA sequencing libraries were prepared according to the manufacturer's instructions (Illumina Truseq stranded mRNA library prep kit). mRNA was purified and fragmented from total RNA (1 μg) using poly-T oligo-attached magnetic beads under two rounds of purification. Cleaved RNA fragments primed with random hexamers were reverse-transcribed into first-strand cDNA using reverse transcriptase, random primers, dUTP in place of dTTP (The incorporation of dUTP quenches the second strand during amplification, because the polymerase does not incorporate past this nucleotide). These cDNA fragments then underwent addition of a single ‘A’ base and subsequent ligation of the adapter. The products were purified and enriched with PCR to create a final strand-specific cDNA library. The quality of the amplified libraries was verified via capillary electrophoresis (Bioanalyzer, Agilent). After qPCR using SYBR Green PCR Master Mix (Applied Biosystems), we combined libraries that index tagged in equimolar amounts in the pool. Cluster generation occurred in the flow cell on the cBot automated cluster generation system (Illumina). Then the flow cell was loaded onto the Next-seq 500 sequencing system (Illumina), and sequencing was performed with a 2 × 150-bp read length.

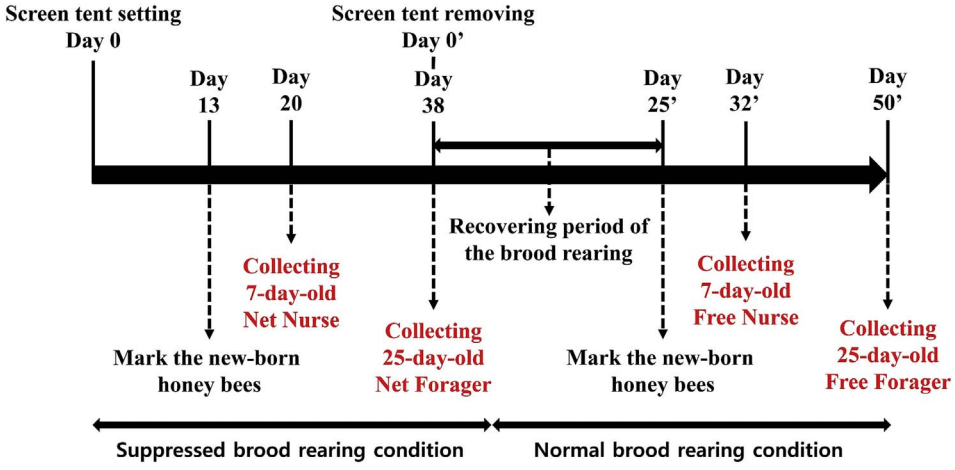

Fig. 1. Honey bee collection scheme. Four active honey bee colonies were placed in a screen tent for complete suppression of brood rearing for a period of 38 days during summer. Thirteen days after the screen tent installation, the last batch of newly hatched honey bees (500 individuals per hive) were marked with paint markers. On days 20 and 38 the marked honey bees were collected as 7-day-old nurse bees (Net Nurse) and 25-day-old forager bees (Net Forager), respectively. The screen tent was removed on day 38 and all the pre-marked bees were eliminated. It took nearly 25 days for brood rearing to recover. On day 25′, the first batch of newly hatched honey bees were marked as before and collected on days 32′ and 50′ as 7-day-old nurse bees (Free Nurse) and 25-day-old forager bees (Free Forager), respectively.

2.3. Transcriptome data analysis

Low quality reads were filtered out according to the following criteria: reads containing > 10% skipped bases (marked as 'N'), reads containing > 40% of bases with quality scores < 20 and reads with an average quality score < 20. Filtered reads were mapped to the honey bee genome analysis data [20] as a reference from BCM-HGSC using the CLC Assembly Cell v4.2 (CLC Bio, Denmark). Gene expression level was measured with Cufflinks v2.1.1 [21] using the gene annotation database of honey bee genome analysis data [20] as a reference from BCM-HGSC. The non-coding gene region was removed via the mask option. Information of this research was submitted to NCBI BioProject (PRJNA386067) and Sequence Read Archive (SRA; SRR5534311, SRR5534312, SRR5534313, SRR5534314)

2.4. GSEA

GSEA was performed to examine critical pathways represented under different conditions [22]. The estimated expression levels of all annotated genes (n = 10,943) were applied to GSEA and then ES values were calculated according to the ranked-ordered gene list. As the predefined gene sets, the Kyoto Encyclopedia of Genes and Genomes (KEGG) information deposited in NCBI Biosystems was analyzed using an in-house script. A total of 132 KEGG pathways were considered, and pathways containing a minimum of 15 genes were evaluated. The significance scores were computed using the 1000 nonparametric permutation test. To get some idea on the overall profiles of enriched pathways in all test comparisons (including both directions) and their possible clustering patterns between either related pathways or between test comparisons, pathways significantly enriched (solely based on nominal p-value of ≤ 0.05) in any of the test comparisons were clustered using 2-way hierarchical clustering. For this, the distances between pathways were calculated based on Euclidean distance and the clustering was completed using linkage methods. The resulting clustering patterns were then visualized using Heatmap3 library in R package (Fig. 2) [23]. Pathways over-represented under each condition with statistical significance [enrichment score (ES) ≥ 0.33 or ≤ -0.35; nominal $p \leq 0.05$; false discovery rate (FDR) q ≤ 0.30] were further identified and characterized in detail. The threshold for the FDR value was set at 0.3, which was slightly higher than the standard value (0.25) [22], in order to identify pathway changes under less stringent conditions. Pair-wise comparison of enriched pathways was mainly conducted for the basic four sets of conditions, which likely occur in natural setting: 1) Free Nurse vs. Free Forager, 2) Free Nurse vs. Net Nurse, 3) Free Forager vs. Net Forager and 4) Net Nurse vs. Net Forager (Table 1 and S Table 1).

3. Results and discussion

3.1. GSEA

To better understand the GSEA results, pathways with a p-value < 0.05 in any of the test conditions were clustered and displayed using a heat map (Fig. 2). Although the heat map result indicated that the pathways did not cluster clearly, notable changes in some pathways induced by labor division and brood rearing status and a few rough clustering patterns among the comparisons were observed. For examples, the comparison of Free Nurse vs. Free Forager was clustered with that of Net Forager vs. Free Forager, suggesting that the physiological changes in both Free Nurse and Net Forage relative to Free Forager are similar each other. Likewise, the reverse comparison of Net Nurse vs. Free Nurse was somewhat closely clustered with that of Free Forager vs. Free Nurse, suggesting that the physiological changes in both Net Nurse and Free Forager relative to Free Nurse are similar each other. Taken together, when the normal labor of both nurses and foragers was suppressed, their overall profiles of pathway representation resembled those of normal foragers and nurses, respectively. Most of these changes were related to genetic information processing and metabolism. Under all two conditions of Free Nurse and Net Forager, remarkable changes in the pathways related to genetic information processing (Protein processing in endoplasmic reticulum) and metabolism (Glycerophospholipid metabolism) were noted compared to Free Forager (actively engaged in foraging).

To investigate the affected pathways in detail, the pathways over-

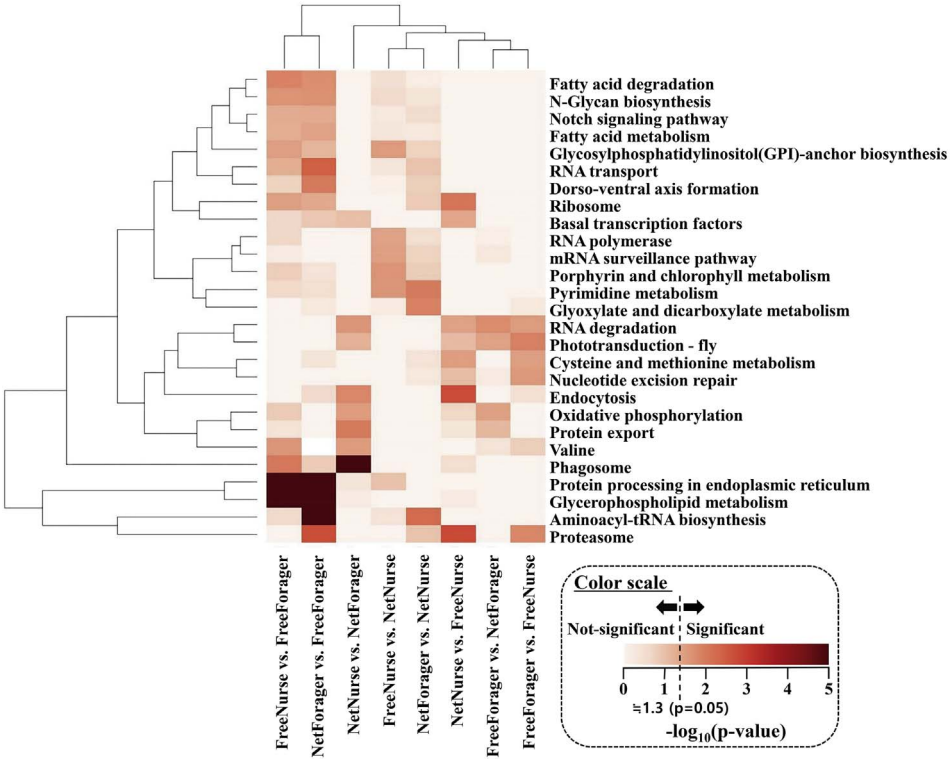

Fig. 2. Heat map illustrating enriched pathways with a statistical significance in each group. Pathways with the nominal p-value ($p \leq 0.05$) in any test comparison were listed and clustered according to the related pathways (Y-axis) and different test comparisons (X-axis). Heat map color scale is based on $-\log_{10}(p$-value).

Table 1
List of statistically significant over-represented pathways identified from GSEA under the normal and brood-rearing suppressed conditions (see S Table 1 for detailed statistical parameters associated with each enriched pathway).

Categories from KEGG pathway[a]		Pathway name	Free Nurse vs. Free Forager		Free Nurse vs. Net Nurse		Free Forager vs. Net Forager		Net Nurse vs. Net Forager	
Main categories	Sub categories		Free Nurse	Free Forager	Free Nurse	Net Nurse	Free Forager	Net Forager	Net Nurse	Net Forager
Metabolism	Lipid metabolism	Glycerophospholipid metabolism	O					O		
		Fatty acid degradation	O					O		
	Glycan biosynthesis and metabolism	*N*-glycan biosynthesis	O					O		
		Glycosylphosphatidylinositol (GPI)-anchor biosynthesis	O							
	Amino acid metabolism	Cysteine and methionine metabolism		O						
		Valine, leucine and isoleucine degradation						O		
	Nucleotide metabolism	Pyrimidine metabolism			O					
	Global and overview map	Fatty acid metabolism						O		
	Energy metabolism	Oxidative phosphorylation							O	
	Carbohydrate metabolism	Glyoxylate and dicarboxylate metabolism								O
Genetic information processing	Folding, sorting and degradation	Protein processing in endoplasmic reticulum	O					O		
		RNA degradation		O		O			O	
		Proteasome				O		O		
		Protein export							O	
	Transcription	RNA polymerase			O					
	Translation	Ribosome	O			O		O		
		RNA transport						O		
		Aminoacyl-tRNA axis formation						O		
	Replication and repair	Nucleotide excision repair		O						
Cellular processes	Transport and catabolism	Phagosome	O						O	
		Endocytosis				O			O	
Organismal system	Development	Dorso-ventral axis formation						O		
Environmental information processing	Signal transduction	Notch signaling pathway						O		

Statistically significant pathways (ES ≥ 0.33; NOM $p \leq 0.05$; FDR q ≤ 0.30) with or without brood rearing being suppressed were listed.
[a] KEGG PATHWAY Database (http://www.kegg.jp/kegg/pathway.html).

represented with statistical significance under each condition (ES ≥ 0.33 or ≤ − 0.35; NOM $p \leq 0.05$; FDR q ≤ 0.30) were summarized and compared in a pairwise manner in Table 1. To observe pathway changes in a more conservative way, a slightly higher threshold for the FDR q value (0.30) was employed than recommended (0.25) [22]. Based on these criteria, a total of 10, 6, 12 and 6 pathways were detected in the pairwise comparisons for Free Nurse vs. Free Forager, Free Nurse vs. Net Nurse, Free Forager vs. Net Forager and Net Nurse vs. Net Forager, respectively (Table 1 and S Table 1); when the FDR q value was set at ≤ 0.25, however, only 6, 2, 11 and 3 pathways were affected in these same respective comparison groups.

3.2. Labor-specific pathways

Six pathways related with either metabolism or genetic information processing were significantly over-represented ($p < 0.03$) in Free Nurse with respect to Free Forager (Table 1 and S Table 1, see Free Nurse vs. Free Forager). Nurse bees synthesize the major protein components of royal jelly in hypopharyngeal glands and secrete the lipid-rich components to the brood. Since the major components of royal jelly are proteins and fatty acids [24], pathways associated with protein biosynthesis and processing (Protein processing in endoplasmic reticulum and Ribosome), lipid metabolism (Glycerophospholipid metabolism and Fatty acid degradation) and glycan biosynthesis and metabolism [*N*-glycan biosynthesis and Glycosylphosphatidylinositol (GPI)-anchor biosynthesis] are likely to be up-regulated in active nurse bees. The fatty acid degradation (mostly β-oxidation) [25] pathway was over-represented in Free Nurse when compared with Free Forager, suggesting that nurse bees may rely more on fatty acid degradation for energy generation. Although up-regulation of the citrate cycle pathway was reported in the brain of nurse bees in a previous study [18], it was not observed in our GSEA (ES score, − 0.342; p value, 0.2857).

The phagosome formation pathway was over-represented in Free Nurse when compared with Free Forager (Table 1, see Free Nurse vs. Free Forager). Since phagocytosis is a main cellular immune pathways [26], the nurse-specific up-regulation of phagosome pathway likely reflects enhanced immune activity in nurses, which is necessary to brood care.

In the Free Forager, up-regulation of the cysteine and methionine metabolism pathway was observed (Table 1 and S Table 1, see Free Nurse vs. Free Forager). Since foragers do not consume a protein diet, they may need to generate sulfur-containing essential amino acids, such as cysteine and methionine, from other amino acids via this pathway to maintain homeostasis. Another more speculative interpretation takes into account the biological function of *S*-adenosyl methionine [20], a major intermediate in the cysteine and methionine metabolism pathway. SAM functions as a coenzyme of remarkable metabolic versatility, thereby playing a main role in DNA and protein methylation [27]. Considering that DNA methylation differences are observed between nurses and foragers [28], differential regulation of the cysteine and methionine metabolism pathway may be associated with labor-specific DNA methylation.

The nucleotide excision repair (NER) pathway was also up-regulated in Free Forager (Table 1, see Free Nurse vs. Free Forager). Since

Table 2
Differentially up-regulated genes in the neuroactive ligand-receptor interaction pathways with fold-change values of > 2 under the normal and brood-rearing suppressed conditions (Free Nurse/Free Forager vs. Net Nurse/Net Forager).

Condition	Gene description	Fold change
Free Nurse/Free Forager	GABA-gated ion channel	13.1
	Serotonin receptor	6.26
	Serotonin receptor	2.76
	Diuretic hormone receptor	2.47
	Adipokinetic hormone receptor	2.10
Free Forager/Free Nurse	PREDICTED: partitioning defective 3 homolog	57.7
	Adenosine receptor	10.2
	NMDA receptor 1	8.13
	Metabotropic glutamate receptor 2	8.00
	Ligand-gated chloride channel homolog 3	7.64
	D2-like dopamine receptor, transcript variant X2	5.12
	Metabotropic GABA-B receptor subtype 3	2.54
	Uncharacterized	2.28
	Dopamine receptor, D1	2.01
Net Nurse/Net Forager	Serotonin receptor 7	10.6
	D2-like dopamine receptor, transcript variant X2	3.46
	Serotonin receptor	3.45
	Cys-loop ligand-gated ion channel subunit 8916	2.52
Net Forager/Net Nurse	Muscarinic acetylcholine receptor DM1	19.2
	Diuretic hormone 31 receptor 1	5.83
	Serotonin receptor	3.93
	Diuretic hormone receptor	3.39
	Metabotropic glutamate receptor 2	2.31

NER is the main DNA repair system against DNA damage by photo-radiation [29], foragers are expected to exhibit enhanced NER to compensate for any possible DNA damage related to radiation.

The RNA degradation pathway was over-represented in Free Forager (Table 1 and S Table 1, see Free Nurse vs. Free Forager). As RNA degradation is a main component in gene expression control, this finding indicates that gene expression occurs relatively more actively in foragers.

In summary, the labor-specific pathways predicted from GSEA reflected the physiologies that can be naturally expected from worker bees based on respective division of labor. This finding further suggests that overall predictions obtained from GSEA are reliable.

3.3. Pathways affected under brood rearing suppression

When the nursing activity of young bees was artificially blocked (i.e., Net Nurse) by confining beehives within a screen tent and their gene-set enrichment profiles were compared with those of active nurses (i.e., Free Nurse; Table 1 and S Table 1, see Free Nurse vs. Net Nurse), the pathways involved in macromolecule degradation (Proteasome and RNA degradation) were up-regulated in Net Nurse. At the same time, protein biosynthesis (Ribosome) was over-represented in Net Nurse. This simultaneous over-representation of pathways associated with both protein degradation and synthesis indicates the active reformation of physiology when normal labor (i.e., nursing) is artificially blocked. Interestingly, the RNA degradation pathway was commonly over-represented in both Net Nurse and Free Forager, when compared to Free Nurse, suggesting that some portion of the nurse bees' physiology is altered and becomes similar to that of foragers under this condition.

When normal foraging was blocked as in the case of Net Forager (Table 1 and S Table 1, see Free Forager vs. Net Forager), bees exhibited over-representation of some common pathways that are typically up-regulated in Free Nurse with respect to Free Forager. These pathways included the glycerophospholipid metabolism, fatty acid degradation, *N*-glycan biosynthesis, protein processing in endoplasmic reticulum and ribosome pathways. This finding again suggests that when the foraging activity of foragers is artificially blocked, their physiology becomes somewhat similar to that of nurses. Taken together, these findings further support that transition of labor is physiologically reversible [9].

In addition, Net Forager exhibited up-regulation of pathways associated with translation (Ribosome, RNA transport and Aminoacyl-tRNA biosynthesis) (Table 1 and S Table 1, see Free Forager vs. Net Forager). Interestingly, the pathway related with protein degradation (Proteasome) was also up-regulated in Net Forager. The simultaneous activation of protein degradation and synthesis observed in Net Forager again suggests that blocking of foraging may induce dynamic reformation of body physiology, as also observed in Net Nurse (Table 1 and S Table 1, see Free Nurse vs. Net Nurse).

The dorso-ventral axis formation pathway was also over-represented in Net Forager with respect to Free Forager (Table 1 and S Table 1, see Free Forager vs. Net Forager). Since dorso-ventral axis formation is a principal procedure in the embryogenic development of the central nervous system in both insects and vertebrates [30], its differential up-regulation in adult bees depending on foraging status is difficult to explain. Nevertheless, its significant over-representation in Net Forager (ES = -0.64; $p = 0.01$) suggests some meaningful physiology changes in adult bee brain when foraging activity is blocked.

The notch signaling pathway was over-represented in Net Forager compared with Free Forager (Table 1 and S Table 1, see Free Forager vs. Net Forager). The notch signaling pathway is a highly conserved pathway across most multicellular organisms that is not only involved in the regulation of cell differentiation during embryogenesis, but also plays a pivotal role in the control of neuronal function and development in the adult brain [31]. Notch signaling is also activated in olfactory receptor neurons of the adult brain of *Drosophila melanogaster* in an odorant-specific fashion, suggesting its role in response to olfactory stimulation [32]. Taken together, the differential regulation of the notch signaling pathway between Net Forager and Free Forager may suggest induced neural plasticity related to the disturbance of labor division status.

3.4. Neuroactive ligand-receptor interaction pathways differentially regulated under different conditions

Although no significant statistical parameters were obtained from GSEA (ES = -0.33, -0.39, -0.34 and -0.27; $p = 0.36$, 0.21, 0.43 and 0.91; q = 0.65, 0.55, 0.69 and 0.44 in pairwise comparisons of Free Nurse vs. Free Forager, Free Nurse vs. Net Nurse, Free Forager vs. Net Forager and Net Nurse vs. Net Forager, respectively), various molecular components in the neuroactive ligand-receptor interaction pathway were investigated, as many neuroactive ligands are involved in physiological responses to stress (e.g., biogenic amines, neuropeptides and metabolic hormones) [33]. Some genes were differentially expressed under different conditions, and those with fold-change values of > 2 were listed in Table 2 and S Table 2.

3.4.1. Genes over-expressed in normal nurses and foragers

The most up-regulated neuroactive ligand-receptor in active nurses (Free Nurse) relative to foragers (Free Forager) was the GABA-gated ion channel (Grd) (13.1 fold). Up-regulation of the GABA-gated ion channel appears to induce overall reduction in neuronal excitability in nurses [34], which would be natural or beneficial to in-hive tasks. In addition, two serotonin receptors were up-regulated in nurses (2.8–6.3 fold), suggesting that stress (or aggression) management between nest mates is more important in nursing state within the confines of the beehive compared with foraging state [35].

Approximately two-fold up-regulation of the adipokinetic hormone (AKH) receptor was observed in nurses. AKH is a typical metabolic hormone produced in the corpora cardiaca and released into the hemolymph to mobilize the main energy source (i.e., trehalose) from the

fat body [33]. Because foragers use the nectar carried in their crops as the sole sugar energy source for flight [36,37] and their fat body is almost completely depleted [14], the AKH-mediated energy mobilization is unlikely to occur in foragers in contrast to nurses. In addition, it is known that almost no AKH is expressed in foragers [38]. Taken together, up-regulation of the AKH receptor in nurses closely reflects differential modes of energy utilization depending on labor division. Given that diuretic hormone receptor (DHR) along with AKH has been suggested to stimulate the release of trehalose and glucose from the fat body [33], the physiological meaning of increased expression of DHR (2.5 fold) in nurses can be explained in the same manner.

The adenosine receptor (AR) was approximately 10-fold over-expressed in Free Forager relative to Free Nurse. Adenosine, through interaction with ARs, is involved in both neurosignal integration and synaptic plasticity modulation (e.g., long-term potentiation and long-term depression) in the brain regions associated with learning and memory, suggesting the role of the AR in cognition control [39]. With this in mind, up-regulation of the AR in foragers relative to nurses suggests that neuromodulation via the AR is enhanced in foragers, which is necessary to boost learning and memory. Recently, the caffeine in nectar has been suggested to enhance honeybee olfactory associative memory by antagonizing AR, thus increasing foraging efficiency [40]. This finding also supports the idea that AR-mediated neuromodulation is relatively more important in foragers and is likely enhanced by over-expression of ARs in foragers.

Consistent with AR, both *N*-methyl-D-aspartate (NMDA) and metabotropic glutamate receptors were approximately 8-fold over-expressed in Free Forager relative to Free Nurse. Glutamate is involved in the regulation of synaptic functions associated with learning and memory via NMDA receptors in the brain of mammals [41]. Likewise, glutamatergic neurons in the honeybee brain were suggested to be involved in processing of long-term olfactory memory [42], and metabotropic glutamate receptors to be required for memory formation [43]. Therefore, significant up-regulation of NMDA and glutamate receptors may indicate enhanced olfactory learning and memory activity, which are both essential in finding and memorizing the location of food sources.

Up-regulation of the LCCH3-type GABA receptor (7.64 fold) was observed in Free Forager as well. Since the inhibitory neuronal transmission mediated by RDL- and LCCH3-type GABA receptors in the honeybee antennal lobes was suggested to play a critical role in olfactory information processing [44], up-regulation of the LCCH-type receptor makes sense in forager bees that are supposed to collect nectar and pollen. Interestingly, a metabotropic GABA-B receptor was over-expressed (2.54 fold) as well. Considering that the GABA-B receptor is involved in the regulation of defensive behaviors of foragers [45], its over-expression in foragers is likely associated with their outdoor tasks.

3.4.2. Genes over-expressed in brood rearing-suppressed nurses and foragers

When beehives were placed inside a screen tent, thereby perturbing the normal labors of worker bees, some genes became commonly over-expressed in both nurses (Net Nurse) and foragers (Net Forager) relative to Free Nurse and Free Forager (Table 3). A similar extent of over-transcription was found for translocator protein (88.2 and 99.9 fold) and allatostatin-A receptor (AAR) (6.8 and 8.8 fold) in Net Nurse and Net Forager, respectively.

Translocator protein (TSPO) is an evolutionarily well-conserved protein family [46]. The primary physiological function of TSPO has been suggested to be the regulation of oxidative stress [46]. After screen tent installation, many foragers found hanging on the screen showed sluggish locomotion and lethargic symptoms (field observation, K. Kim) and exhibited higher mortality compared to normal colonies. Considering that *Drosophila* TSPO mediates apoptosis, reduces longevity and regulates neurodegeneration [47], the over-expression of TSPO in both nurses and foragers may be related to control of various forms of cellular stress affecting worker bees after screen tent installation. However, the physiological function of TSPO in honey bees remains to be elucidated.

Table 3
Up-regulated transcripts of the neuroactive ligand-receptor interaction pathway both in the Net Nurse and Forager.

Gene description	Fold change	
	Net Nurse/Free Nurse	Net Forager/Free Forager
Translocator protein	88.2	99.9
Allatostatin-A receptor	6.78	8.78
Prolactin releasing peptide receptor-like	2.93	8.13
GABA-gated ion channel	1.06	14.7
NMDA receptor 1	11.3	1.05
Metabotropic GABA-B receptor subtype 1	2.07	1.33
Metabotropic glutamate receptor	1.26	2.10

The allatostatin-A receptor (AAR) was 6.8- and 8.8-fold over-expressed in both Net Nurse and Net Forager, respectively. It has been postulated that the allatostatin-A type neuropeptide is expressed in the honey bee CC and involved in energy mobilization control, particularly under conditions of chronic and acute nutritional stress [33]. Bees were expected to be under acute nutritional stress under the screen tent condition, as food was restricted, and thus AAR-mediated regulation of energy mobilization would be necessary in both Net Nurse and Net Forager. In addition, the up-regulation of the AAR may be involved in the control of aggressiveness. As the aggressiveness of honey bee is related to juvenile hormone (JH) titer in worker bees [48] and allatostatin-A regulates JH release from the CC [49], the over-expression of AAR may be required to reduce the aggressiveness of bees confined inside the screen tent, thereby minimizing stress levels among nest mates.

A GABA-R gene (*Grd*) was selectively over-expressed (14.7 fold) in Net Forager relative to Free Forager. Overexpression of the Grd GABA-gated ion channel likely reduces the neuronal excitability in Net Forager [34], which would be beneficial to foragers under perturbation of normal behavior in terms of adaptation or stress reduction. A similar trend in *Grd* over-transcription was also observed in Free Nurse relative to Free Forager (Table 2), suggesting that enhanced expression of the Grd GABA-gated ion channel is not related to worker bee age, but is likely task-dependent.

Significant up-regulation of the NMDA receptor (11.3 fold) in Net Nurse relative to Free Nurse, which is also a representative response in Free Forager (Table 2), likely indicates overall enhancement of olfactory learning and memory in nurse bees and possible labor transition to foraging when their normal nursing activity is abolished. This selective up-regulation of the NMDA receptor solely in Net Nurse but not in Net Forager further suggests another aspect of premature physiological transition from nurse bees to forager bees when labor division becomes ambiguous.

A metabotropic GABA-B receptor was approximately two-fold over-expressed in Net Nurse relative to Free Nurse. As the metabotropic GABA-B receptor is involved in defensive behavior regulation of foragers [45], its over-expression in nurses when normal nursing activity was obstructed may indicate a sign of premature labor transition.

A metabotropic glutamate receptor was approximately two-fold over-expressed in Net Forager relative to Free Forager. Assuming that metabotropic glutamate receptors are required for memory formation [43], this may suggest the need for enhanced learning and memory when foraging-related tasks are completely blocked.

4. Conclusions

Comparison of the pathways predicted from GSEA and the expression profiles of some genes in the neuroactive ligand-receptor interaction pathway between normal nurses and foragers enabled the identification of labor-specific pathways, which was expected based upon the behavior and physiology of worker bees with different tasks. However, when active beehives were placed in a screen tent, thereby blocking normal labor and eventually leading to complete suppression of brood rearing, pathways reflecting dramatic changes in physiology and behaviors were identified. Some genes associated with the regulation of neuronal excitability, cellular and nutritional stress and aggressiveness were differentially expressed depending upon labor state (nursing vs. foraging) and in the presence of absence of brood rearing suppression. Under brood rearing suppression, worker bees exhibited reduced neuronal excitability and aggressiveness, which likely facilitates the management of in-hive stress under unfavorable conditions.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.ygeno.2017.08.004.

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